Biochimica et Biophysica Acta, 480 (1977) 390—402
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BBA 68031

PURIFICATION AND PROPERTIES OF POLYPHOSPHOINOSITIDE PHOSPHOMONOESTERASE FROM RAT BRAIN

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(Received July 19th, 1976)

Summary

1. On subcellular fractionation of rat brain homogenate, polyphosphoinositide phosphomonoesterase activity was greater in the cytosol than the membranous fractions.

2. The enzyme was purified from the cytosol by column chromatography on DEAE-cellulose, calcium phosphate gel and Sephadex G-100.

3. The final preparation of the enzyme showed a 430-fold purification over the whole homogenate and appeared to be homogeneous since it gave a single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and on isoelectric focusing. The enzyme has a relatively low molecular weight and an isoelectric point of 6.8.

4. The phosphatase showed a high affinity for triphosphoinositide. Without added Mg^{2+}, the K_m was 25 μM and V was 33 μmol P_i released/min/mg protein.

5. The enzyme hydrolysed diphosphoinositide at a slower rate than triphosphoinositide. In the presence of 10 mM Mg^{2+}, the K_m values for triphosphoinositide and diphosphoinositide were 5 μM and 25 μM respectively and V was the same for each substrate.

6. Both Mg^{2+} and Ca^{2+} activated the enzyme. While Ca^{2+} produced maximum activation at 100 μM, a much higher concentration of Mg^{2+} (10 mM) was required to elicit comparable activation. The enzyme did not show an absolute requirement for Mg^{2+} or Ca^{2+} as it exhibited low activity in the presence of 0.5 mM EDTA or EGTA.

7. The phosphatase showed maximum activity between 7.4 and 7.6. A drop in pH to 7.0 inactivated it almost completely, whereas an increase in pH to 8.0 halved the activity.

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Introduction

Polyphosphoinositides * are found in myelin [1,2] although they also occur in other subcellular organelles of brain [3], membranes of other mammalian tissues and certain micro-organisms [4]. These phospholipids are negatively charged and interact with Ca^{2+} [5,6] and other membrane components rather strongly [1,7,8]. Experiments with radioactive phosphate show a very high turnover of the monoester phosphate groups of these phospholipids [1,2,7,9, 10] and an alteration in their metabolism would influence their interaction with Ca^{2+} and other components of membranes. Such an alteration could induce changes in membrane structure and function [7,11]. Neomycin is toxic to inner ear tissues and also decreases labelling of triphosphoinositide in vivo by $^{32}P_i$ in these tissues [12]. The drug also competitively inhibits calcium ion binding to homogenates of cochleae. Recently, Hawthorne [13] and Michell [14] have discussed various possibilities as to how polyphosphoinositide metabolism could be related to physiological events in vivo.

It has been shown that polyphosphoinositides disappear very readily post mortem [2,15] and the activities of synthetic enzymes are considerably less than those of the hydrolytic enzymes [11] suggesting a strict regulation of the latter in vivo. Such control could be by compartmentalization of the hydrolytic enzymes and/or their lipid substrates, or by metabolite inhibition. Recent evidence indicates the asymmetric distribution of phosphoinositides in red blood cell membranes [16] and also of enzymes concerned with their metabolism [6,10,17].

Phosphatidylinositol kinase is a membrane-bound enzyme whereas [19] diphosphoinositide kinase is mainly found in the soluble fraction of brain [11]. Polyphosphoinositide phosphomonoesterase and phosphodiesterase have been reported as largely soluble enzymes in brain [19]. In kidney, both soluble and membrane-bound activity was found [20,21,22]. Brain triphosphoinositide phosphomonoesterase is stimulated by a component of the pH 5 supernatant. Taking this into accound, Sheltawy et al. [23] considered that the monoesterase was more likely to be associated with plasma membranes than with the soluble fraction [24].

Since the hydrolytic enzymes are highly active and probably play a significant role in the turnover of polyphosphoinositides in vivo, attempts have been made to study their mechanism of action. Some properties of phosphomonoesterase in crude preparations from brain [23,24] and kidney [20,21] and with partially purified preparations from brain [25,26] have been described. However, no information is yet available about the properties of the pure enzyme.

In this communication, we describe a procedure to purify polyphosphoinositide phosphomonoesterase from the cytosol of rat brain and illustrate some of its properties.

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* Since there is not yet an agreed systematic nomenclature, triphosphoinositide is here used as a more convenient name for phosphatidylinositol 4,5-bisphosphate and diphosphoinositide for phosphatidylinositol 4-phosphate. The two phospholipids together are referred to as polyphosphoinositides.

Materials and Methods

Preparation of polyphosphoinositides. Bovine brains were frozen in liquid nitrogen immediately after their removal from animals in the slaughter house and brought to the laboratory on ice. A crude diphosphoinositide fraction was prepared by the method of Folch [27] and individual phospholipids separated from it by column chromatography on DEAE-cellulose (Whatman DE 52, microgranular) exactly as described by Hendrickson and Ballou [28]. Pooled lipid fractions were concentrated by rotary evaporation, dialysed against 0.5 mM EDTA, lyophilized, resuspended in a small volume of distilled water and stored at −25° C.

Preparation of calcium phosphate gel. Calcium phosphate gel was prepared according to the procedure of Keilin and Hartree [29].

Measurement of polyphosphoinositide phosphomonoesterase activity. The reaction mixture contained 45 mM Tris · HCl buffer, pH 7.2, 1 mM $MgCl_2$, 1 mM NaF, 0.5% (w/v) bovine serum albumin (fatty acid-free, Sigma, London), 3 mM cysteine (preparated fresh on the day of use, pH 7.2), 1.0 mM each of triphosphoinositide (sodium salt) and cetyltrimethylammonium bromide and 5—50 μg of the enzyme protein in a total volume of 0.15 ml. The reactants were equilibrated at 37° C for 5 min and the reaction was initiated by the addition of an equimolar mixture of triphosphoinositide and cetyltrimethylammonium bromide, continued for 15 min and stopped by the addition of 0.025 ml of 10% (w/v) bovine serum albumin and 0.15 ml of ice-cold trichloroacetic acid (5%, w/v). The mixture was centrifuged for 15 min in a bench centrifuge and the clear supernatant was analysed for P_i by the method of Chen et al. [30].

Measurement of conductivity. Conductivity of the samples was measured with a conductivity meter (Radiometer, Copenhagen). Tris concentration in fractions obtained from the DEAE-cellulose column was estimated from a conductivity plot of standard solutions containing Tris · HCl buffer, pH 7.4 (10—750 mM).

Estimation of protein. Proteins were precipitated with ice-cold trichloroacetic acid (5% w/v), dissolved in 1 N NaOH and analysed by the method of Lowry et al. [31]. Standards containing 0.25 to 50 μg of bovine serum albumin were treated similarly and analysed for protein.

Subcellular fractionation of rat brain. Male rats weighing about 250 g were anesthetized with ether (clinical grade) and decapitated. Whole brain was quickly removed, washed with ice-cold 0.32 M sucrose and homogenized by hand in 10 volumes of 0.32 M sucrose. The homogenate was centrifuged at 10 000 × *g* for 20 min and the resulting supernatant was centrifuged further for 60 min at 105 000 × *g*. The pellets of both centrifugations (P_1 and P_2) were suspended in 0.32 M sucrose. These subfractions were analysed for enzyme activity and protein. The supernatant from the 105 000 × *g* centrifugation was used as source of enzyme.

Purification of polyphosphoinositide phosphomonoesterase from rat brain supernatant

Column chromatography was performed at room temperature unless otherwise noted.

Chromatography on DEAE-cellulose. DEAE-cellulose (Whatman DE 52, microgranular, pre-swollen) was washed and equilibrated in 20 mM Tris · HCl buffer, pH 7.4 and a column (2.25 × 35 cm) was packed using the same buffer. The brain supernatant (about 150 mg protein) was made 20 mM with respect to Tris · HCl (pH 7.4) and applied to the column. The enzyme was eluted with a continuous Tris gradient made by mixing equal volumes (275 ml each) of 20 mM and 250 mM Tris · HCl buffer, pH 7.4 and finally applying 75 ml of 250 mM Tris · HCl buffer, pH 7.4. Fractions (12 ml) of eluate were collected and analysed for conductivity, absorbance at 280 nm and for enzyme activity. Storage of fractions overnight at 2—4° C or for longer periods of time at —25° C did not result in a loss of activity.

Chromatography on calcium phosphate gel-cellulose. Calcium phosphate gel (880 mg dry wt.) was mixed with a slurry of 10 g cellulose powder (Whatman, standard grade, ashless) in distilled water. The mixture was poured into a column (3.5 cm diameter and packed to a height of 5.0 cm with distilled water at a flow rate of 2 ml/min maintained by a peristaltic pump (Watson-Marlow Ltd., Falmouth). The column was first washed with about 100 ml of distilled water and then equilibrated with 75 ml of 75 mM Tris · HCl buffer, pH 7.4. Pooled fractions from the DEAE-cellulose column (about 10 mg protein) were applied and the effluent (F_1) was collected. The column was then washed with 75 ml of 75 mM Tris · HCl buffer, pH 7.4 (effluent F_2). The enzyme was eluted with 75 ml each of 50, 100, 250 and 500 mM potassium phosphate buffer, pH 8.0, collecting the effluent in separate fractions, F_3, F_4, F_5 and F_6 respectively. Fractions were poured into dialysis sacs which had previously been boiled for 3 h and thoroughly rinsed with distilled water. The fractions were dialysed against 5 l of cold distilled water (changed twice in 5 h) and then against 20 l of 5 mM Tris · HCl buffer, pH 7.4, changed periodically. Dialysis was carried out in the cold room at 2—4° C. Prolonged dialysis against distilled water inactivated the enzyme, presumably by the drop in pH, because (a) dialysis against the buffer for 72 h did not harm the enzyme and (b) a drop in pH from 7.4 to 6.5 almost completely inactivated it (Fig. 8).

Chromatography on Sephadex G-100. Sephadex G-100 (fine mesh, Pharmacia, Sweden) was suspended in distilled water and allowed to swell for 2 days at 2—4° C. About 12 h prior to its use, the gel was allowed to warm up at room temperature. Water was decanted and a thick slurry was poured into a column (2.25 × 36 cm) and packed by running distilled water through slowly (0.5 ml/min) keeping a head of 28 cm of water. A column volume (about 250 ml) of 20 mM Tris · HCl buffer, pH 7.4 was passed through to equilibrate the column. The enzyme fraction (see Results) obtained from the DEAE-cellulose and calcium phosphate gel columns was lyophilized, resuspended in 10 ml of 20 mM Tris · HCl buffer, pH 7.4 and applied to the column. The enzyme was eluted with 250 ml of the same buffer, collecting 4-ml fractions. The fractions were analysed for protein (*A* at 280 nm) and enzyme activity as described above.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The enzyme preparation from the Sephadex column was dialyzed against distilled water overnight and rotary-evaporated to dryness. The subsequent treatment and electrophoresis followed the method of Betts and Mayer [32], as did the staining of the gel for protein. The stained gel was scanned at 600 nm using a Unicam SP 1800 UV spectrophotometer with scanning densitometer.

Results

Subcellular distribution of polyphosphoinositide phosphomonoesterase activity in rat brain

On crude subcellular fractionation of rat brain homogenate, about 70% of polyphosphoinositide phosphomonoesterase activity was associated with the membranous fractions and about 55—60% activity was found in the soluble fraction (Table I). The recovery of enzyme activity was always more than 100%, suggesting the presence of an inhibitor in either the soluble or membrane fractions. Higher recoveries of this enzyme on subcellular fractionation have been observed by others [7,21]. When the activity in the 10 000 × *g* pellet was further subfractionated on a continuous sucrose gradient in a B XIV zonal rotor (M.S.E., London), most of the activity was associated with the synaptosomal fraction. On disruption of synaptosomes, about 65—70% of the activity was recovered in the synaptosomal cytosol while the remaining 30—35% was still associated with the membranes. The enzyme activity in the whole homogenate of rat brain was similar to that reported by others [24].

Chromatography on DEAE-cellulose

Soluble proteins of rat brain were resolved into many protein peaks of variable sizes (Fig. 1). The enzyme activity was well separated from the major protein peak (tubes 31—37) and eluted in peak 2 (tubes 37—44) and peak 3 (tubes 45—55). The material in peak 2 contained more endogenous P_i (high blanks) than that in peak 3 (low blanks). When the soluble fraction was chromatographed on a small wide column (3.5 × 15 cm) of similar bed volume under similar conditions, the elution profile of proteins was not much changed, but the enzyme eluted in one broad peak which was not well separated from major protein peak.

Chromatography on calcium phosphate gel

When the proteins contained in peak 2 fractions from the first column were applied to the calcium phosphate gel column, almost all were retained (Fig. 2a,

TABLE I

SUBCELLULAR DISTRIBUTION OF POLYPHOSPHOINOSITIDE PHOSPHOMONOESTERASE ACTIVITY IN RAT BRAIN

The tissue was homogenised in 0.32 M sucrose (10%, w/v) and fractions were prepared by differential centrifugation as described in the text. Whole homogenate of rat brain contained 77.2 ± 8.3 mg protein/g tissue and showed triphosphoinositide phosphomonoesterase activity of 0.66 ± 0.25 μmol P_i released/mg protein/h.

Subcellular fraction	Protein (% whole homogenate)	Polyphosphoinositide phosphomonoesterase (% whole homogenate)
$Pellet_1$, 10 000 × *g*, 10 min	63 ± 6	58 ± 6
$Pellet_2$, 105 000 × *g*, 60 min	12 ± 2	14 ± 3
Supernatant, 105 000 × *g*, 60 min	18 ± 2	54 ± 23
Recovery	95 ± 6	126 ± 28

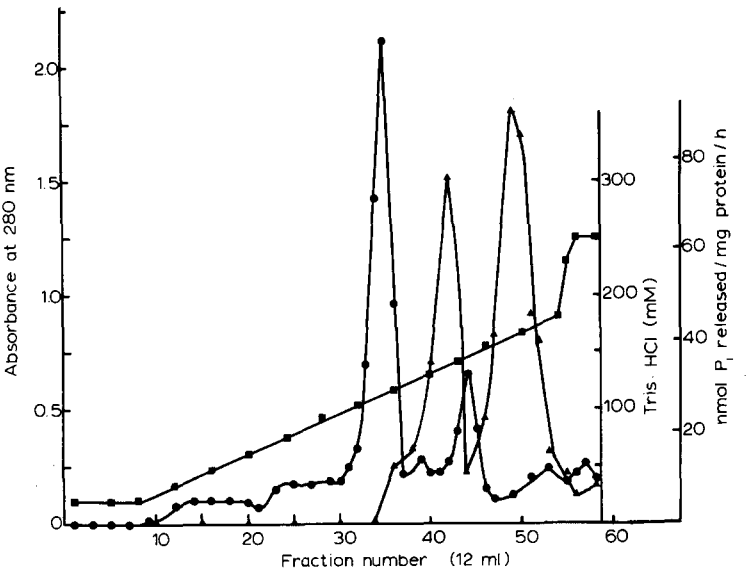

Fig. 1. Polyphosphoinositide phosphatase purification on a DEAE-cellulose column. Brain supernatant was applied to the column as described in the text. Protein (*A* 280 nm) ●, Tris · HCl concentration ■, enzyme activity ▲.

F_1 and F_2). Application of 75 ml each of 50 and 100 mM potassium phosphate buffer, pH 8.0 eluted most of the extraneous proteins (F_3 and F_4 respectively) while the enzyme activity was still retained. The enzyme activity was first eluted with 250 mM potassium phosphate and was completely eluted with the application of 500 mM potassium phosphate buffer (F_5 and F_6). A 30—50 fold purification over the DEAE-cellulose fraction was always achieved in F_6.

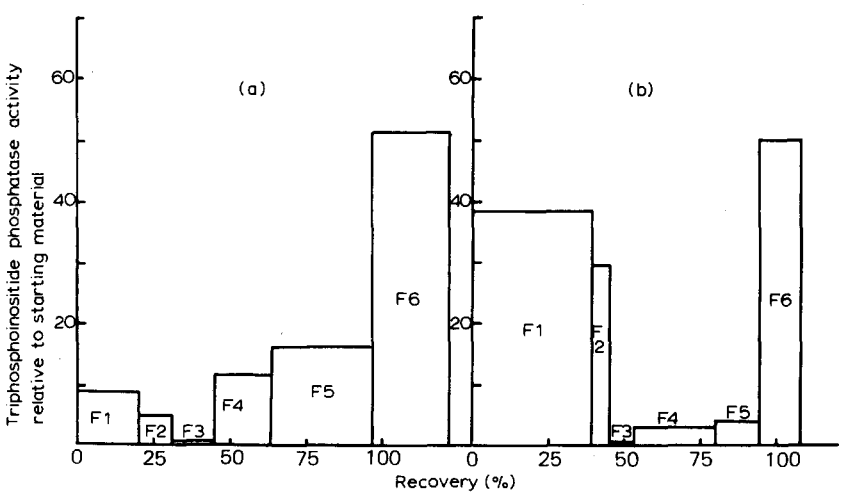

Fig. 2. Chromatography on calcium phosphate gel-cellulose. Details are given in the text. (a) Peak 2 from Fig. 1 applied to column. (b) Peak 3 from Fig. 1 applied. Protein distributions were as follows (% protein applied): Fig. 2a F_1, 2.5; F_2, 2.0; F_3, 39.6; F_4, 18.0; F_5, 6.7; F_6, 1.1; recovery 69.9%. Fig. 2b F_1, 3.0; F_2, 2.0; F_3, 35.6; F_4, 16.9; F_5, 5.2; F_6, 2.7; recovery 65.4%.

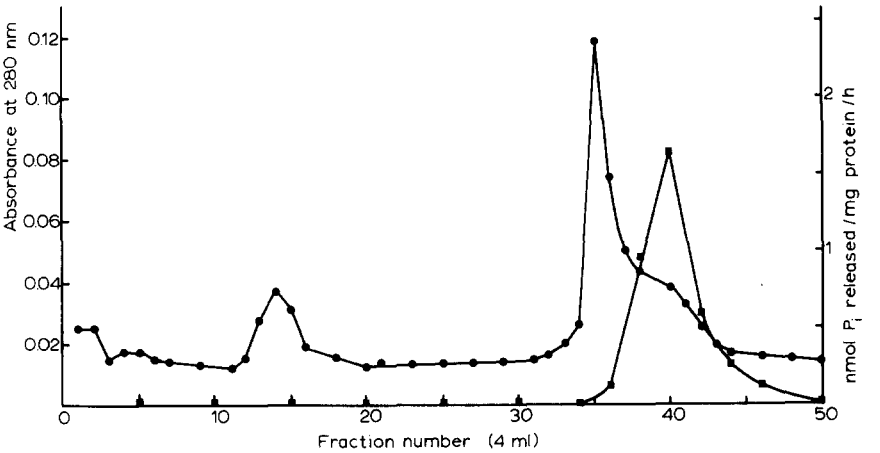

Fig. 3. Chromatography on Sephadex G-100 column. Fraction 6 (Fig. 2a) from the calcium phosphate column was lyophilized after dialysis and applied to the Sephadex G-100. Details are given in the text. Protein ●, enzyme activity ■.

When the proteins from peak 3 fractions (Fig. 1) were chromatographed under exactly similar conditions, about 50% of the enzyme activity simply passed through the column while extraneous proteins along with a portion of enzyme activity were retained (Fig. 2b). Extraneous proteins were once again eluted with 50 and 100 mM potassium phosphate buffer and only application of 500 mM potassium phosphate buffer could elute the remaining enzyme activity.

Chromatography on Sephadex G-100

Fraction 6 obtained from the calcium phosphate gel column of Fig. 2a was resolved further into three protein peaks by chromatography on Sephadex G-100 (Fig. 3). A small protein peak appeared after the void volume, a second major peak appeared later and had a clear shoulder (tubes 38—45) which contained all the polyphosphoinositide phosphomonoesterase activity. About 3-fold purification over the DEAE-cellulose and calcium phosphate gel fraction was achieved. The elution pattern of enzyme activity was exactly similar when fraction 6 from the DEAE-cellulose column of Fig. 2b was separated on Sephadex G-100.

Purity of the final enzyme preparation

The results in Table II show the stepwise purification of polyphosphoinositide phosphomonoesterase from the rat brain homogenate. The final preparation contained about 5% of the starting activity and showed a purification of about 430-fold over the tissue homogenate. Similar preparations of enzyme were obtained consistently in a number of trials.

When the final preparation of enzyme was subjected to dodecyl sulphate-polyacrylamide gel electrophoresis, it gave a single band and appeared to be of relatively low molecular weight (Fig. 4). The purity of the enzyme preparation was also checked by isoelectric focussing on a 5% polyacrylamide gel. The enzyme migrated as a single band with a pI value of 6.8.

TABLE II

PURIFICATION OF POLYPHOSPHOINOSITIDE PHOSPHOMONOESTERASE FROM RAT BRAIN SUPERNATANT

Fraction	Protein (mg)	Specific activity (μmol P_i/mg protein/h)	Recovery (%)	Purification (fold)
Whole homogenate	3498	1.03	100.0	1.0
Supernatant (105 000 X g, 60 min)	660	2.47	45.2	2.4
DEAE-cellulose	154	6.34	27.1	6.2
Calcium phosphate gel	3.42	164.93	15.7	160.1
Sephadex G-100	0.38	443.60	4.7	430.7

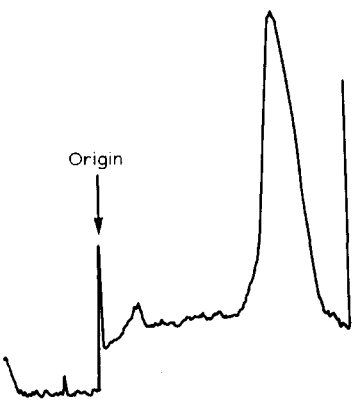

Fig. 4. Densitometer tracing of dodecyl sulphate-polyacrylamide gel after electrophoresis of the purified enzyme and staining with Coomassie Blue.

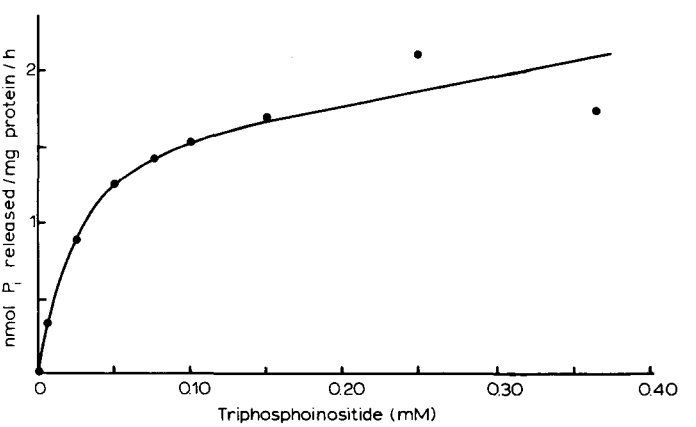

Fig. 5. Polyphosphoinositide phosphatase activity as a function of substrate concentration. The reaction mixture containg 45 mM Tris · HCl buffer, pH 7.4, 5 μg purified enzyme and variable triphosphoinositide concentration in a final volume of 0.15 ml. After equilibration for 5 min at 37°C the reaction was started by adding the enzyme. The reaction was stopped after 15 min as described in the text for assay of P_i.

Properties of polyphosphoinositide phosphomonoesterase

On incubation with triphosphoinositide in a buffered medium (pH 7.4), the enzyme released P_i at a constant rate for 15 min. The rate of triphosphoinositide hydrolysis was proportional to enzyme concentration over the range 0—15 μg per ml, at least.

The enzyme activity increased as a function of triphosphoinositide concentration and reached a maximum at about 0.25 mM (Fig. 5). In the presence of the detergent cetyltrimethylammonium bromide, the rate of hydrolysis continued to increase in a linear fashion to a much higher concentration of triphosphoinositide i.e. 0.15 mM and apparently a much higher level of the lipid was required to saturate the enzyme (unpublished data). A Lineweaver-Burk plot of the data in Fig. 5 showed that the K_m for triphosphoinositide was 25 μM and V was 2 mmoles P_i released/mg/h. Over almost the whole range of concentrations used in Fig. 5 the enzyme would be presented with single molecules of triphosphoinositide, since the critical micellar concentration is 0.3 mM (Pickard, M.R. and Hawthorne, J.N., unpublished work).

The enzyme did not show an absolute requirement for Mg^{2+} or Ca^{2+} as it hydrolysed triphosphoinositide in the presence of 0.5 mM EDTA or EGTA (Fig. 6). Both Mg^{2+} (Fig. 6a) and Ca^{2+} (Fig. 6b) activated the enzyme, but their mode of action appeared to be different. While low concentrations of Ca^{2+} maximally activated the enzyme, relatively higher concentrations of Mg^{2+} were required to elicit comparable activity. Higher concentrations of Ca^{2+} reduced the activation, which was almost abolished at 25 mM Ca^{2+}. In contrast, excess

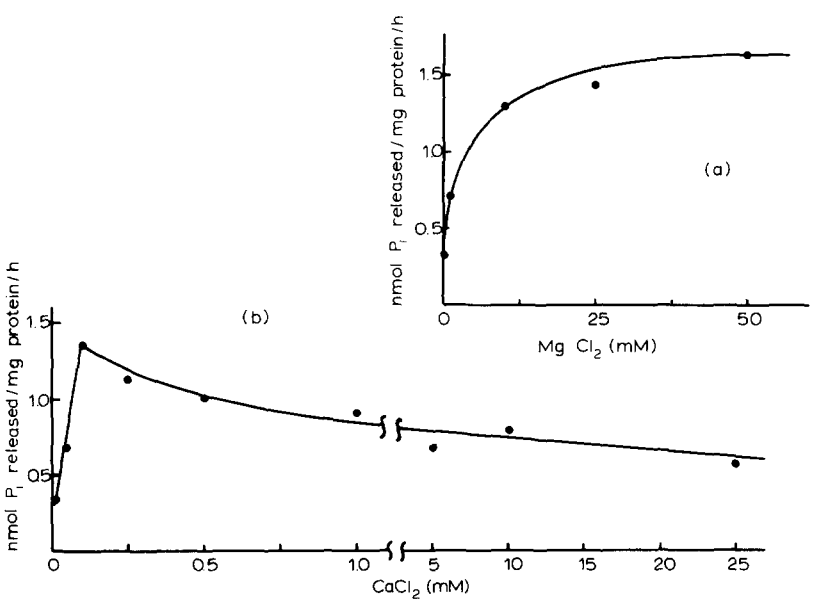

Fig. 6. Effects of (a) Mg^{2+} concentration and (b) Ca^{2+} concentration on polyphosphoinositide phosphatase. The assay mixture contained 45 mM Tris · HCl buffer, pH 7.4, 5 μg purified enzyme and 0.25 mM triphosphoinositide sodium salt in a final volume of 0.15 ml. Other details followed the legend to Fig. 5. Assays without added divalent cations contained (Fig. 6a) 0.5 mM EDTA and (Fig. 6b) 0.5 mM EGTA (ethylene glycol-bis-(β-amino ethyl ether)*N,N'*-tetraacetic acid) respectively.

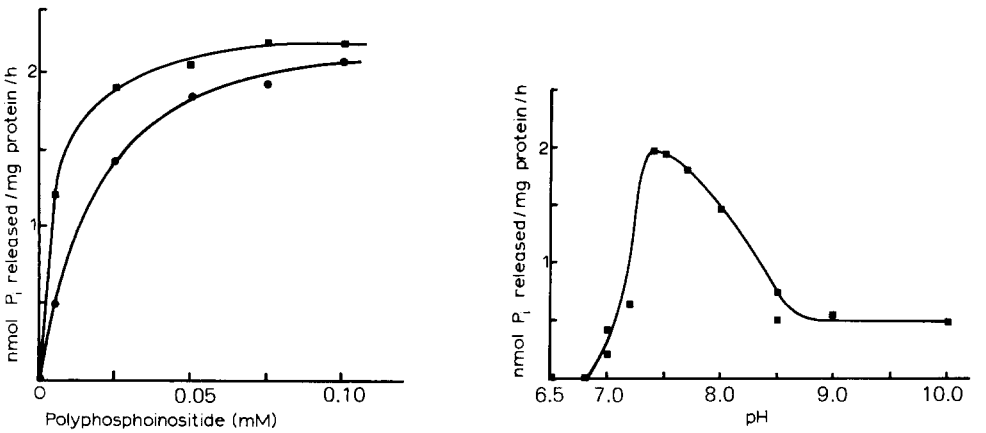

Fig. 7. Hydrolysis of triphosphoinositide and diphosphoinositide by the purified enzyme. The reaction mixtures contained enzyme and buffer as described in the legend to Fig. 5, together with 10 mM $MgCl_2$ and triphosphoinositide (■) or diphosphoinositide (●) in the concentrations shown.

Fig. 8. Influence of pH on polyphosphoinositide phosphatase. The assay mixture contained 45 mM buffer, 0.25 mM triphosphoinositide, 10 mM $MgCl_2$, 0.25 mM cetyltrimethylammonium bromide and 5 μg purified enzyme in a total volume of 0.15 ml. The reaction was started by the addition of mixture containing triphosphoinositide and cetyltrimethylammonium bromide, after equilibration for 5 min at 37°C. Buffers were as follows: pH 6.5—7.0, imidazole/HCl; pH 7.0—8.5, Tris · HCl; pH 7.5—10.0, glycine/NaOH.

Mg^{2+} did not diminish the maximum activation. In the presence of 10 mM Mg^{2+}, the K_m of the enzyme for triphosphoinositide was reduced from 25 μM to 5 μM, however, the V was not changed.

The enzyme also hydrolyzed diphosphoinositide yielding P_i and phosphatidylinositol (Fig. 7). The K_m for diphosphoinositide was 25 μM in the presence of 10 mM Mg^{2+}, compared with a figure of 5 μM for triphosphoinositide. V was the same with both substrates.

The enzyme was most active between pH 7.4 and 7.6 and was almost inactivated by the drop in pH to 7.0 (Fig. 8). An increase in pH to 8.0, however, reduced the enzyme activity by only 50%.

A change in incubation temperature from 10 to 20°C did not alter the

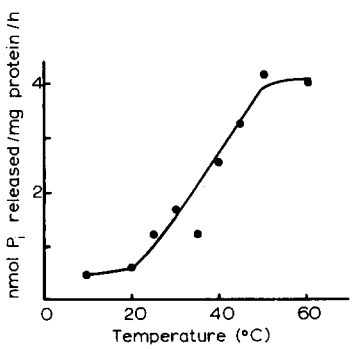

Fig. 9. Effect of temperature on polyphosphoinositide phosphatase. The assay mixture (with 45 mM Tris · HCl buffer, pH 7.4) is described in the legend to Fig. 8.

enzyme activity. At higher temperatures the reaction rate was proportional to temperature with maximum hydrolysis of triphosphoinositide at 50° C (Fig. 9). Further increase in temperature to 60° C did not alter the activity and incubation at 75° C inactivated the enzyme.

Discussion

The results of this study are consistent with the earlier observations on brain [19,24] and kidney [21] that triphosphoinositide phosphomonoesterase activity was partly soluble and partly associated with membranes. Whether the two activities are different from each other is not yet clear. An attempt was therefore made to purify the enzyme from the soluble fraction of rat brain and a method has been worked out which consistently yields a homogeneous preparation of the enzyme.

The height of the DEAE-cellulose column appeared to be critical for the effective separation of the enzyme from other soluble proteins. Furthermore, the use of a tall narrow column resolved the enzyme activity into two peaks which appeared to be distinct from one another. The P_i content of enzyme in peak 2 was higher than that of the enzyme in peak 3 and the elution pattern of the two proteins from the calcium phosphate gel column differed considerably (Figs. 2a and 2b). Whether the pattern was different because of the higher ionic strength of fractions in peak 2 or because of differences in the enzyme proteins is not yet clear. However, after chromatography on calcium phosphate gel the proteins behaved identically on Sephadex G-100, sodium dodecyl sulphate-polyacrylamide gel electrophoresis and isoelectric focussing. Maeno and Greengard [33] reported recently the purification of phosphoprotein phosphatase from the cytoplasmic fraction of rat brain by column chromatography on DEAE-cellulose. Their protein elution profile was very similar to that reported here and phosphoprotein phosphatase activity eluted in two peaks which followed the major protein peak. Their further studies showed that the enzymes in the two phosphatase peaks were different and could have originated from different cell types. It is possible that the two peaks of polyphosphoinositide phosphomonoesterase activity originated from different cell types.

At physiological pH the enzyme would be negatively charged (pI 6.8) so that interaction with the strongly anionic triphosphoinositide would be minimal. Triphosphoinositide has a high affinity for calcium ions [5,6] and probably exists in brain as the calcium/magnesium salt [2]. It might be expected that optimum activation by calcium ions would occur when the triphosphoinositide was neutralized fully. Fig. 6b shows that maximum activity was obtained when only 100 μM Ca^{2+} was present in an assay medium containing 250 μM triphosphoinositide. In contrast to magnesium ions, higher concentrations of calcium ions were inhibitory, in confirmation of earlier work with a partly purified enzyme [25]. In this earlier work, maximum activation was only obtained when a considerable excess of calcium or magnesium was present. The difference may be accounted for by other proteins in the enzyme preparation used by Dawson and Thompson [25].

Intracellular concentrations of calcium (less than 1 μM) and magnesium ions (about 8 mM) in brain tissue are well below the optima for activation of poly-

phosphoinositide phosphomonoesterase (Fig. 6). This will contribute to the stability of triphosphoinositide in vivo. Rapid post mortem hydrolysis by the enzyme is well known, and a rise in intracellular Ca^{2+} may be responsible. Another activator may be the pH 5 supernatant factor of Sheltawy et al. [23]. Calculations from the homogenate activity of Table I show that the enzyme could hydrolyse all the triphosphoinositide of brain in about 1 min.

While intracellular calcium and magnesium ion concentrations may not be sufficient for appreciable hydrolysis of triphosphoinositide in vivo, it is still possible that local influx of extracellular calcium could be important in this process, as was suggested some years ago [1]. Rapid breakdown of triphosphoinositide in red cell membranes can be initiated by Ca^{2+} [10].

Talwalkar and Lester [34] suggested that the absolute concentrations of polyphosphoinositides in yeast cells directly reflected the intracellular ATP concentration. This may also apply to mammalian cells. Given sufficient ATP, all the membrane phosphatidylinositol of the pig erythrocyte, for instance [6], can be converted into diphosphoinositide or triphosphoinositide. The rapid loss of these lipids from brain post mortem may also reflect lack of ATP.

The critical micellar concentration for triphosphoinositide as the sodium salt is 0.3 mM. For most of the substrate concentrations used in Fig. 5 therefore, the enzyme will be presented with single molecules rather than micelles of triphosphoinositide and the K_m of 25 μM will be a true one. A higher V could be obtained in the presence of the detergent cetyltrimethylammonium bromide, suggesting that micelle formation when no detergent is available prevents the enzyme from being saturated with substrate. Sheltawy et al. [23] quote a K_m of 143 μM for the enzyme of brain homogenate in the presence of activating K^+.

The purified enzyme hydrolysed both diphosphoinositide and triphosphoinositide, in keeping with work from this laboratory on the enzyme in kidney [21]. We were unable to detect triphosphoinositide phosphodiesterase activity in the purified enzyme from brain (unpublished observations).

Acknowledgements

This work was supported by a grant from the Multiple Sclerosis Society of Great Britain. The authors are indebted to Dr. Bruce Middleton for helpful discussions and advice during the course of this work. The help of Miss S.A. Betts and Dr. J.G. Bowen (Department of Cancer Research) with dodecyl sulphate-polyacrylamide gel electrophoresis and isoelectric focusing respectively is very much appreciated.

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